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Effects of Temperature, pH, Salinity, and Inorganic Nitrogen on the Rate of Ammonium Oxidation by Nitrifiers Isolated from Wetland Environments

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16. ABSTRACT

Ammonium-oxidizing bacteria were examined in two wetland environments, a freshwater marsh and an estuarine bay, during a 2-year period. Two predominant types were consistently isolated, one from each environment. Both isolates were identified as species of Nitrosomonas. Using a closed culture, high cell density assay, the effects of temperature, pH, salinity, Na^+, K^+, nitrite, nitrate, and ammonium concentrations on ammonium oxidation were determined. Maximum activity was observed for the freshwater isolate at 35°C, pH 8.5, salinities of 0.3 to 0.5% Na^+ and K^+, and ammonium concentrations greater than 0.5 g/l. For the estuarine isolate, maximum activity was observed at 40°C, pH 8.0, salinities of 0.5 to 1.0%, 1.0% Na^+ and K^+, and 0.2 g/l ammonium. The estuarine isolate had a Na^+ requirement which could be partially substituted by the K^+, suggesting that the organism is a true estuarine bacterium. Nitrite inhibited both isolates at concentrations greater than 5 mg/l, whereas nitrate had no significant effect on either isolate.

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MICROBIAL ECOLOGY

Effects of Temperature, pH, Salinity, and Inorganic Nitrogen on the Rate of Ammonium Oxidation by Nitrifiers Isolated from Wetland Environments

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Abstract. Ammonium-oxidizing bacteria were examined in two wetland environments, a freshwater marsh and an estuarine bay, during a 2-year period. Two predominant types were consistently isolated, one from each environment. Both isolates were identified as species of *Nitrosomonas*. Using a closed culture, high cell density assay, the effects of temperature, pH, salinity, Na^+, K^+, nitrite, nitrate, and ammonium concentrations on ammonium oxidation were determined. Maximum activity was observed for the freshwater isolate at 35°C, pH 8.5, salinities of 0.3 to 0.5% Na^+ and K^+, and ammonium concentrations greater than 0.5 g/l. For the estuarine isolate, maximum activity was observed at 40°C, pH 8.0, salinities of 0.5 to 1.0%, 1.0% Na^+ and K^+, and 0.2 g/l ammonium. The estuarine isolate had a Na^+ requirement which could be partially substituted by the K^+, suggesting that the organism is a true estuarine bacterium. Nitrite inhibited both isolates at concentrations greater than 5 mg/l, whereas nitrate had no significant effect on either isolate.

In the aquatic environment, transformation of nitrogen and its recycling are extremely important to the productivity of the system. Nitrification is that phase of the nitrogen cycle where ammonium is oxidized to nitrite and nitrate. It is a microbial process, mediated primarily by a group of gram-negative chemolithotrophic bacteria within the family Nitrobacteraceae (1, 19). Although considerable attention has been given to nitrifiers from the agricultural and sewage treatment approach, only a few studies (5, 12, 23, 24) have attempted to investigate the process of nitrification in coastal environments.

Ammonium oxidation in agricultural soils and sewage has been shown to be affected by a number of factors (8, 9) including temperature (10), pH (14), salinity (13), and inorganic nitrogen (2). However, information concerning the effects of these various parameters on nitrifiers from nonagricultural wetland environments is lacking. It was,

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therefore, the purpose of this investigation to (a) isolate strains of ammonium oxidizers from two wetlands, a freshwater marsh and an estuarine bay, which represent the two most divergent environmental types along the coastal wetland continuum; and (b) examine the effects of temperature, pH, salinity, nitrite, nitrate, and ammonium on the rate of ammonium oxidation by the bacteria in axenic culture.

Materials and Methods

Sampling

The sampling areas chosen for this study represented two divergent environments on the coastal wetland continuum: a freshwater marsh near Lac des Allemands, Louisiana, located in the uppermost region of the drainage basin of the Barataria Bay estuary, and a shallow estuarine bay in East Bay, Pensacola, Florida. The freshwater marsh sediments, high in organics with little clay content, were obtained from a cypress and tupelo swamp where water depths varied from 1.0 to 0.1 m. The study area and plots have been described in detail by Conners and Day (7). The estuarine sediments, lower in organics with a high sand content, were obtained from inside and outside of a sea-grass (*Ruppia maritima* L.) bed where water depths varied from 1.5 to 0.5 m. The study area has been described in several available but unpublished manuscripts (J. K. Adams and N. S. Schomer, Masters Theses, The University of West Florida, Pensacola, FL). Following a survey of the number of ammonium oxidizers measured by the most probable number (MPN) technique (15) in representative samples from each environment, the aerobic sediment/water interface was found to be the most populated. Fifty samples from each aquatic environment were collected and examined. Using a sterile spatula and a cylindrical polyvinyl chloride corer (20 × 2 cm), samples of the aerobic sediment/water interface were taken during the summer and spring months in 1977 and 1978, placed in sterile petri dishes on ice (4°C), and returned to the laboratory within 48 hours for analysis.

Enumeration of Ammonium Oxidizers

Using the five-tube series MPN, the number of ammonium oxidizers in each sediment was determined as described by Matulewich et al. (15).

Isolation of Nitrifiers

A modification of the media described by Watson (20) and Schmidt et al. (18) was used for enrichment of ammonium oxidizers: $(NH_4)_2SO_4$–1.5 g/l, $MgSO_4 \cdot 7H_2O$–0.2 g/l, $CaCl_2$–0.02 g/l, K_2HPO_4–0.114 g/l, Fe-EDTA (77 mg $FeSO_4 \cdot 7H_2O$ + 103 mg NaEDTA in 50 ml distilled water)–1.0 ml/l, $Na_2MoO_4 \cdot 2H_2O$–1.0 μg/l, $MnCl_2 \cdot 4H_2O$–2 μg/l, $CoCl_2 \cdot 6H_2O$–2.0 μg/l, $CuSO_4 \cdot 5H_2O$–20.0μg/l, $ZnSO_4 \cdot 7H_2O$–100.0 μg/l, $CaCO_3$–2.0 g/l, and phenol red–0.5 mg/l. One gram of each sediment was added to 50 ml of medium. The salinity of the estuarine medium was adjusted with Instant Ocean Synthetic Sea Salts (Aquarium Systems, Inc., Eastland, OH) to 0.8%. Each 125 ml flask was incubated at 30°C and shaken (90 rpm) on a gyrator shaker (Model 62, New Brunswick Scientific Co., New Brunswick, NJ) for 14 days. Then 1 ml of the culture was inoculated into fresh medium and incubated under the same conditions for an additional 14 days. Also, 10 ml of this culture was inoculated into a 550 ml reaction vessel of a chemostat (Benchtop Bioflow Chemostat, New Brunswick Scientific Co., New Brunswick, NJ). The reaction vessel was equipped with an automatic pH controller and the pH was maintained at 7.5 ± 0.5 by the automatic addition of 10% K_2CO_3. Cultures were vigorously aerated by forcing air through a 20 cm cotton-packed column and agitated by a magnetic stirring bar. The flow rate was adjusted so that there was a 50% dilution per day. The medium used in the chemostat was identical to that previously described except that it contained no $CaCO_3$ or phenol red indicator. Cultures were allowed to grow under these conditions for 14 days. Usually at this stage the ratio of heterotrophic to autotrophic cells was 1:100–1:1000. Once this ratio was achieved, the cultures were serially diluted and inoculated into 50 ml of the original medium in 125 ml Erlenmeyer flasks. Following inoculation, the cultures were incubated for 2 to 3 weeks under the conditions previously described. Cultures were checked for the

presence of contaminants by cultural methods and by direct microscopic observation. The cells were concentrated by centrifugation and examined under a phase contrast microscope. For cultural examination, 1 ml was inoculated into nutrient broth, thioglycollate broth, and trypticase soy broth. If no growth of heterotrophs was observed in the nutrient media, and if no different morphological types were observed microscopically, the cultures were considered axenic. Ammonium oxidizer cultures were maintained by serial transfer of 1 ml into 50 ml of fresh medium every 14 days.

Identification

Actively growing cells were prepared by the method of Watson (21) and observed through a Zeiss Standard 17 phase contrast microscope.

Cells for electron microscopy were grown in Erlenmeyer flasks containing one liter of $CaCO_3$ free medium. The flasks were incubated at 30°C on a shaker and received pH adjustments twice daily to pH 8.0 with sterile 10% K_2CO_3 for 14 days. Cells were fixed and embedded using a modified method of Watson (21). Cells were fixed in 3% glutaraldehyde in the ammonium oxidizer medium for 2 hours at 4°C, then pelleted by centrifugation at 10,000 × *g* at 4°C, washed three times in 0.05 M phosphate buffer, pH 7.5, resuspended in 1% OsO_4 in the same buffer, and kept at 4°C overnight. Cells were then pelleted by centrifugation, washed twice in buffer, and enrobed in 3% Noble agar (Difco). The agar was cut into small blocks which were placed in distilled water and then dehydrated by a series of ethanol solutions up to 100%, placed into 100% propylene oxide, and then embedded in Epon-812. Sections were cut with glass knives and stained with lead citrate and uranyl acetate. Electron micrographs were taken using a Philips EM 201 transmission electron microscope.

Suspensions of cells grown as described for electron microscopy were taken during the exponential phase of growth. The cells were pelleted by centrifugation at 10,000 × *g* for 15 min, washed twice in substrate free medium, and resuspended in the same medium at pH 7.8. An oxidized minus dithionite-reduced spectrum was recorded by a Cary 14 double-beam spectrophotometer at room temperature.

Closed Culture Studies

The procedure for the closed culture test consisted of a series of 16 × 150 mm culture tubes containing 9 ml of ammonium oxidizer medium under various conditions. The tubes were inoculated with 1 ml of a standard inoculum which was prepared by growing the isolates in 200 ml of $CaCO_3$ free medium in 500 ml flasks. The flasks were incubated at 30°C on a shaker at 100 rpm for 5 days. The cultures received pH adjustments to pH 8.0 with 10% K_2CO_3 twice daily. The cultures were then filtered through 0.22 μm Nucleopore filters (Nucleopore Corp. Pleasanton, CA), washed twice with sterile medium, and resuspended in 100 ml of sterile medium. This served as the standard inoculum for all closed culture tests and contained approximately 1×10^7 cells/ml. After inoculation, the tubes were incubated at 30°C at 30 rpm (Model TC-5 tube rotator, New Brunswick Scientific Co., New Brunswick, NJ) for 24 hours. The pH was checked and nitrite concentration in each tube was then assayed using the colormetric method described by Bendschneider and Robinson (3). All tubes were prepared in triplicate and all test series were performed in duplicate.

The temperature series consisted of incubation at 5 to 50°C. The pH series was prepared by adjusting the medium to the proper pH (3.0 to 10.0) with either 0.1 N HCl or 0.1 NaOH. The medium in this series contained no $CaCO_3$ but was identical in all other respects. The salinity series was prepared by adjusting the salinities of the medium (0.0 to 3.0%) with Instant Ocean Synthetic Sea Salts. Concentrations of Na^+ and K^+ (0.0 to 5.0%) were prepared by the addition of NaCl and KCl to Na^+ and K^+ free basal medium. The ammonium series (0.0 to 2.0 g/l ammonium) was prepared using an ammonium free basal medium and adjusting the concentration with $(NH_4)_2SO_4$. Nitrite concentration was adjusted using KNO_2 to give levels of 0.0 to 500.0 mg/l NO_2^--N. Nitrate was adjusting using KNO_3 at concentrations of 0.0 to 2.0 g/l NO_3^--N.

Statistical Analysis

Using the standard statistical program, SAS (SAS Institute, Inc., Raleigh, NC), an analysis of variance was performed and a significant effect was established if the calculated *F* exceeded the probability value at the 95% level of confidence.

Results

The average number of ammonium oxidizers in the samples examined using the MPN method was 330 cells/g wet weight in the estuarine sediments and 440 cells/g wet weight in the freshwater sediments.

From the environmental samples examined only two distinctly different types of ammonium-oxidizing bacteria were consistently isolated, one from each environment. The estuarine isolate was a gram-negative coccus, 0.9 to 1.2 μm in diameter, which occurred singly or occasionally in short chains. They were motile with a single polar flagellum. The freshwater isolate was a gram-negative rod, 1.0 μm in diameter by 1.8 to 2.5 μm in length. The cells had pointed ends with prominent connections between dividing cells. The cells occurred singly or in short chains of two or three interconnected cells and were nonmotile. Neither isolate was able to oxidize nitrite to nitrate.

Electron microscopy showed that both isolates possessed extensive cytomembrane systems which were peripherally located. Although two or more cells of the freshwater isolate were often interconnected, no separating cross walls were visible. Oxidized minus dithionite-reduced absorption spectra of living cells were characterized by peaks at 600, 552, 522, 440, and 423 nm for the estuarine isolate and 600, 552, 522, 465, and 423 nm for the freshwater isolate.

The temperature range for both isolates was wide with significant ammonium oxidation occurring at most temperatures examined (Fig. 1). The estuarine isolate exhibited optimum activity at 40°C, whereas the freshwater isolate was most active at 35°C. The estuarine isolate had a pH range of pH 6.5 to 10.0 with an optimum at pH 8.0, while the freshwater isolate showed activity at pH 6.0 to 10.0 with an optimum at pH 8.5 (Fig. 1). Both isolates were able to grow over a wide range of salinities (Fig. 2). The estuarine isolate showed the greatest activity between 0.5 and 1.0%, whereas the freshwater isolate showed optimum activity at 0.3 to 0.5%. At 0.0% salinity, activity of the estuarine isolate declined 95%. Although the freshwater isolate did not grow well at 0.0%, it retained 60% of its activity at this salinity. Neither strain was active at a salinity of 3.0%. The effects of Na^+ and K^+ concentrations on activity showed that the freshwater isolate oxidized ammonium equally well in both 0.5% NaCl and KCl. The estuarine isolate had an optimum of 0.75 to 1.0% NaCl and was unable to grow in a medium without Na^+ or K^+. The K^+ could partially substitute for the Na^+ as noted by some activity in medium containing KCl alone. However, at 1.0% KCl, only 25% of the maximum activity was achieved.

The effects of inorganic nitrogen (ammonium, nitrite, and nitrate) are shown in Fig. 3. The estuarine isolate had an optimum ammonium requirement of 0.2 g/l NH_4^+-N with reduction of activity at higher concentrations. The freshwater isolate showed a wider range of ammonium concentration for activity, with levels of 0.5 g/l NH_4^+-N and greater giving optimum activity. No inhibition of activity was noted at the higher concentrations of ammonium. Nitrite proved to be inhibitory to the estuarine isolate at greater than 5.0 mg/l NO_2^--N, and although the freshwater isolate was also inhibited at this concentration, its activity was less affected. In contrast, nitrate had no significant effect on the activity of either isolate at up to 2.0 g/l NO_3^--N.

Discussion

Ammonium-oxidizing bacteria are classified according to their cell shape and intracytoplasmic membrane arrangements (6, 16, 21, 22). Although the current

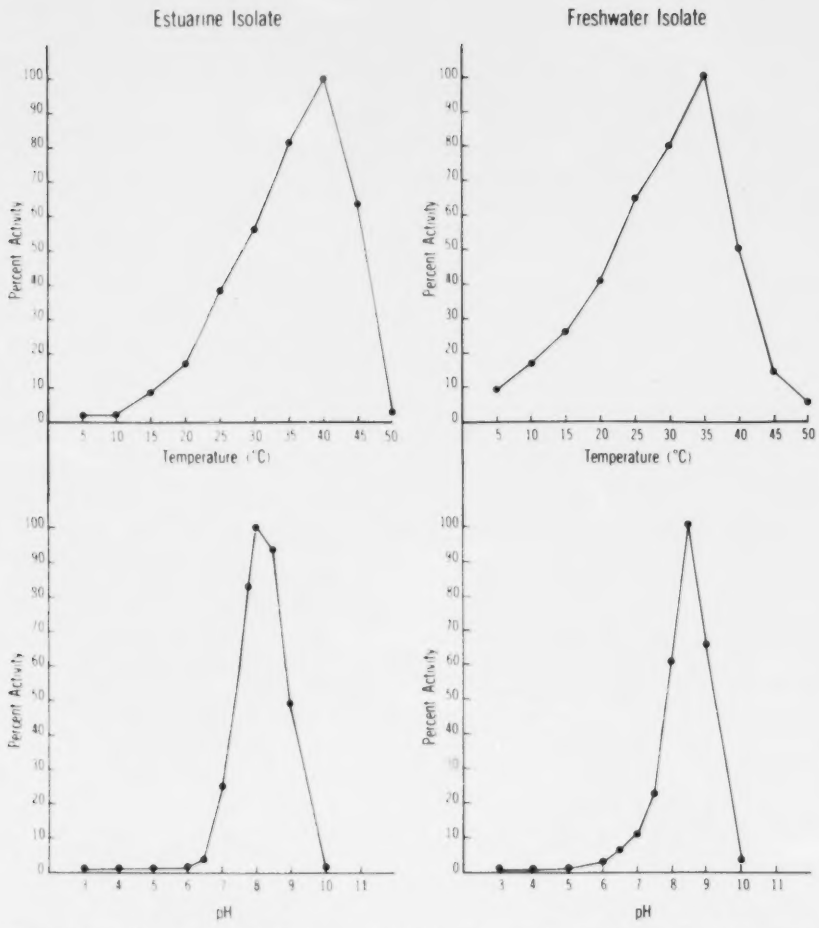

Fig. 1. Effects of temperature and pH on ammonium oxidation by two aquatic nitrifiers. Activity is expressed as the percent of the highest nitrite value obtained under optimum conditions for each isolate.

technique of immunofluorescence has also been applied as a useful taxonomic tool (4), the systematics have not been completely developed for the nitrifiers. When the existing taxonomic scheme in *Bergey's Manual of Determinative Bacteriology* (6) was used, both isolates were placed in the genus *Nitrosomonas*. Although the organisms exhibited characteristics not entirely typical of *Nitrosomonas*, they were placed in this genus to avoid taxonomic confusion. The effects of temperature and pH on the ammonium oxidizer isolates were generally similar to those reported in the literature, although their temperature optima were slightly higher than the 25° to 30°C reported in other studies (1, 17). The elevated optima may be a reflection of the warmer waters from which the organisms were isolated, as in the summer months these waters often exceed 30°C.

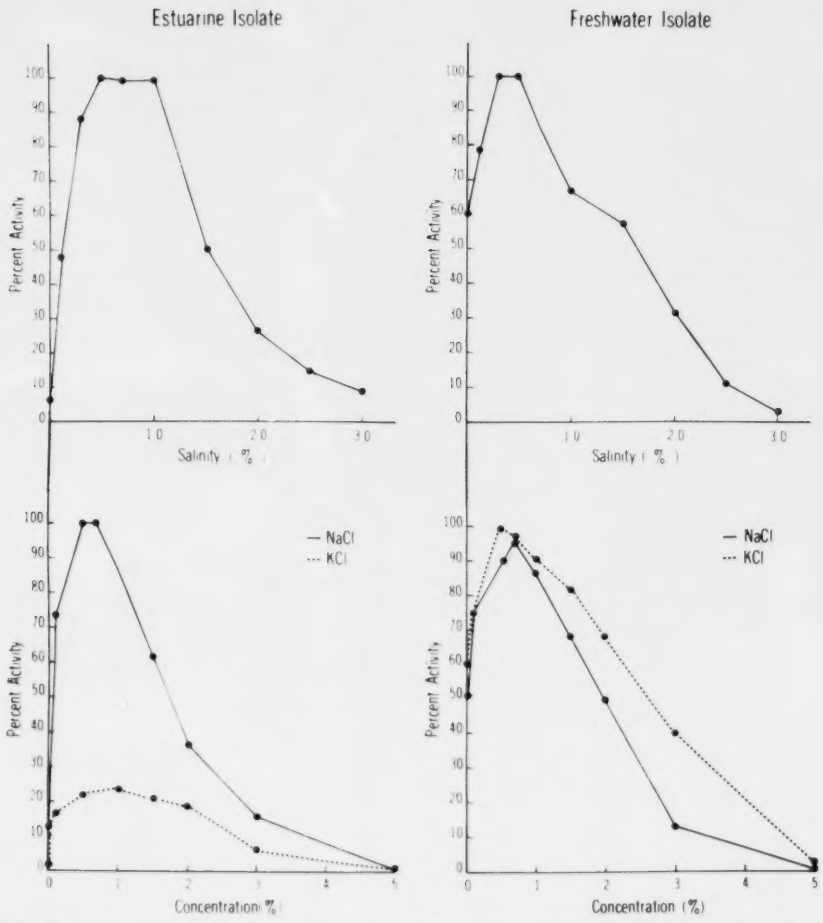

Fig. 2. Effects of salinity, Na^+, and K^+ on ammonium oxidation by two aquatic nitrifiers. Activity is expressed as the percent of the highest nitrite value obtained under optimum conditions for each isolate.

Ammonium oxidation by both isolates was significantly reduced at temperatures below 30°C, suggesting that temperature plays an important role in regulating nitrification in wetland environment. Similarly, the pH optima of 8.0 and 8.5 were well within previously reported pH ranges for other nitrifiers (1, 14, 17), and activity was significantly reduced above and below the optima. Since the pHs of these waters and sediments vary over a wide range (pH 6.3 to 8.5) (11), pH is likely an important factor in regulating ammonium oxidation in these environments.

Salinity and Na^+ and K^+ concentrations affected the activity of both isolates, although the effects of salinity on the freshwater isolate are not especially relevant since there is little salinity change in the freshwater marsh. The estuarine strain exhibited a Na^+ requirement of 0.75 to 1.0% Na^+ for optimum activity and lost all activity at 0.0%

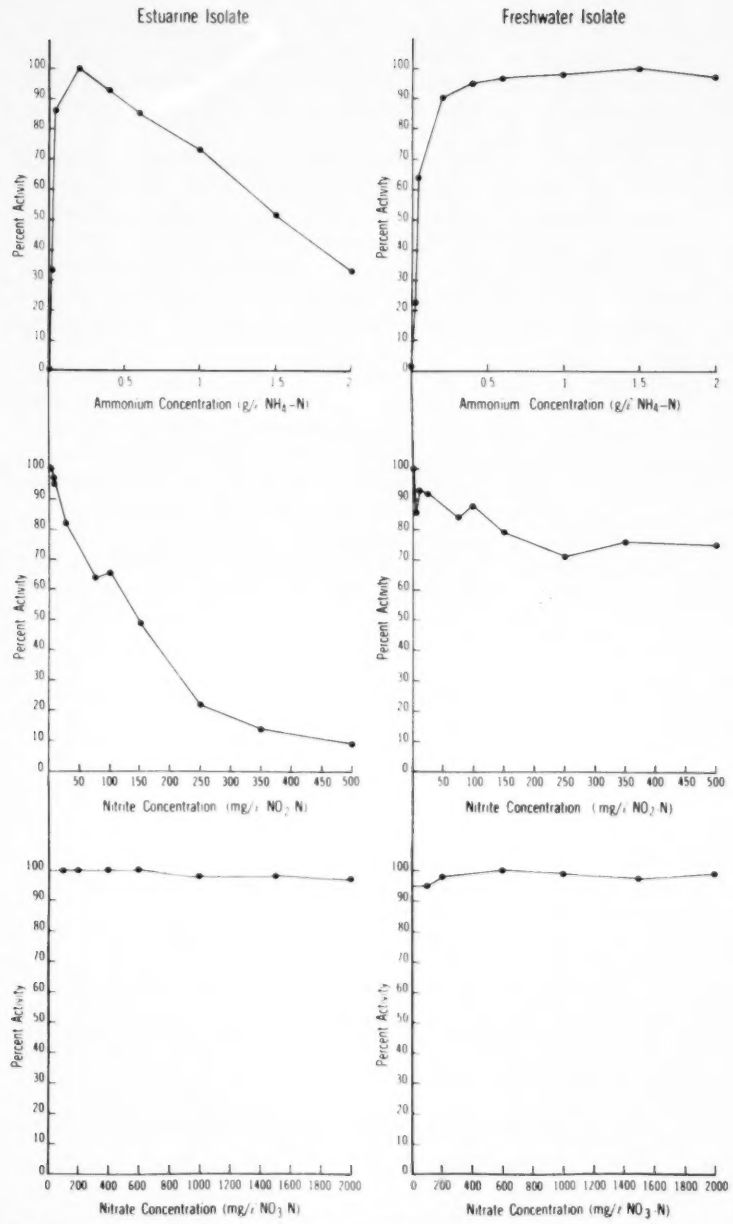

Fig. 3. Effects of inorganic nitrogen, NH_4^+, NO_2^-, and NO_3^- on ammonium oxidation by two aquatic nitrifiers. Activity is expressed as the percent of the highest nitrite value obtained under optimum conditions for each isolate.

Na^+. The K^+ ion could only partially substitute for the Na^+ requirement with 25% of the activity at 1.0% K^+. Because of the salinity requirements, the organism may be considered a true estuarine bacterium. The salinity of this estuary fluctuates during times of heavy rainfall and may fall below 0.5%. When this occurs, ammonium oxidation by the bacterium may be severely reduced.

The response of the isolates in axenic culture to inorganic nitrogen reflects a pattern similar to that noted in soil studies (2). Nitrate had no significant effect on either isolate, although certain concentrations of ammonium and nitrite were inhibitory, especially to the estuarine strain. It has been shown that the inhibitory effects of ammonium and nitrite are dependent on the concentrations of nonionized ammonia (NH_3) and nonionized nitrous acid (HNO_2), and the concentrations of these forms are a function of pH (2). Clearly, the toxicity of these inorganic nitrogen compounds is dependent on environmental pH.

It was interesting to note that the estuarine strain showed considerably more inhibition to ammonium and nitrite than did the freshwater strain. That the freshwater sediments contain far more ammonium and nitrite than the estuarine sediments by an order of magnitude (11) may be relevant to the more tolerant response of the freshwater isolate. Obviously, interpretation of axenic culture responses to environmental parameters must be done with care because of inherent limitations of the assay and the isolation technique. However, with these limitations in mind, the data suggest that ammonium oxidation by isolates from wetlands is influenced by pH and temperature and that estuarine strains may be more sensitive to and affected by salinity, ammonium, and nitrite than those from freshwaters.

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